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E D I T O R I A L

OUR MOST CRITICAL NEED

NEVER before in our memory has the profession of pharmacy been besieged with so many problems coming as they have in recent weeks, one after another, with both a speed and a complexity which must surely stagger those who have the responsibility of doing something about them. If our profession were sufficiently well organized to deal effectively with these problems, the task would be great enough to stagger even the most indefatigable worker but one cannot help but feel sorry for the staff at our Washington headquarters striving as they must without proper support from the profession, and, indeed, with many local and splinter groups going off in all directions, often in a manner which defeats any really effective effort.

We are presently paying the price for several decades of error and poor judgment on the part of those who constitute the profession as well as those who have presumed to give it guidance. Some few have decried these errors and have done so consistently but they have been "voices in the wilderness" drowned out by those who through complacency and inertia would and did allow things to drift into our present state of affairs. One cannot think of a less enviable position than that of those few who today are trying to represent the profession and save it from those who seek to destroy both its professional and economic status.

We have written in this same vein before but never has it been more evident that the profession of pharmacy just is not organized in the United States. It seems quite clear to anyone who assesses our problems objectively that we shall never succeed in solving them until the pharmacists of America belong to a strong national organization with each and everyone giving his or her full support as a member. This membership must be at the county, state, and national levels with complete vertical integration, a single dues structure, and all of the other attributes which characterize the professional organ-

izations of our fellow practitioners in medicine and dentistry. Had this been the pattern in the organization of pharmacy over the past several decades, most of our current problems would not exist.

As we view it, the question is not whether we need such a closely-knit, well-integrated structure in pharmacy but whether, indeed, we can achieve it and how soon. Many pharmacists do not belong to their own county organization; still larger numbers do not belong to the state association; and only about one in four belongs to the American Pharmaceutical Association. To make matters still worse, there is rarely any agreement on policy or program at the county, state, and national level.

The time has surely come when we in pharmacy must begin "calling a spade a spade." We must see those so-called leaders at the county or state level who will have no part of the A. Ph. A. or integration for what they are; namely, those who put personal glory and accomplishment above that of the profession. We wish to state clearly and with all the emphasis at our command that, in our honest opinion, pharmacy will never achieve the recognition and position which is its due until it achieves an organizational plan which makes sense, and a membership which is truly representative of the profession as a whole. Much of that which we presently accept in our disorganized effort is entirely senseless to the objective thinker. The time has come for some drastic changes; in fact, it is long overdue. The question which should trouble all pharmacists and trouble them greatly is whether we can do that which must be done in time. It is our firm conviction that this question overrides all others in its importance and that it should command our immediate attention.

L. F. Tice

EVALUATION OF DRUGS AFFECTING NEURO-MUSCULAR TRANSMISSION *

By William A. Davis

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THE story of the drugs which affect neuromuscular transmission is one of the most interesting, complex, involved developments in pharmacology. It involves black magic in the jungles of the Amazon, historic studies in physiology, and dangerous tests of drugs on human beings. It is not a simple story nor one in which we yet know all the answers for, due to the complexities of the body, it involves not only nerves and skeletal muscle but also brain and smooth muscle and enzyme systems. This paper is to deal with ***methods*** for evaluating the effect of compounds which affect the transmission of nerve impulses to skeletal muscles.

Historical Background

Like so many other things in physiology, our basic ideas on neuromuscular transmission came from Claude Bernard (1). About 1850, he was interested in proving the independent excitability of muscular tissue as distinct from nerves. He had some curare and knew that animals could be completely paralyzed by it but that their muscles would still respond to an electric stimulus after they were paralyzed. He then performed a series of experiments which are classic and which may be summarized as follows:

I. A pithed frog was prepared in such a way that the blood vessels to one leg were tied, and the sciatic nerves to both legs could be stimulated. Such stimulation of the nerves produced contraction of the muscles of both legs before curare was given, but only of the leg where the blood vessels had been ligated after curare was given. This proved that the action of curare was peripheral since ligation of the blood vessel had protected the leg from the action of the drug.

II. Stimulation of the leg muscles by direct electrical shocks caused contractions in both legs, both before and after curare. This

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* Presented at the Seminar on *Current Methods of Drug Evaluation,* February 1959, Philadelphia College of Pharmacy and Science, Philadelphia 4, Pa.

proved that the drug had not caused paralysis by injury of the muscle itself.

III. When a section of the nerve in the leg where the muscle was still able to contract was exposed to curare, stimulation of the nerve above the exposed area still caused contraction of the muscle. This proved that curare did not stop the nerve from transmitting impulses.

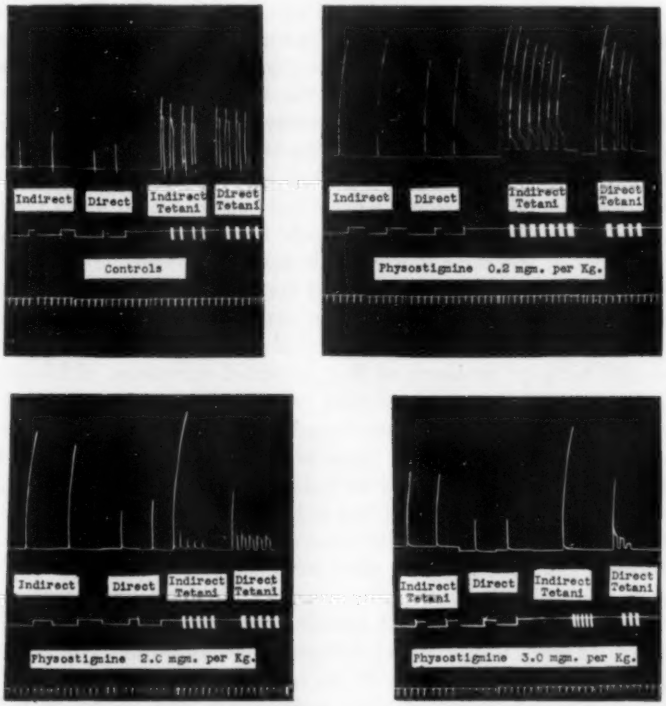

FIG. 1 *

Cat, 2.5 Kg. Anesthesia—95% ethyl alcohol (7.5 cc. per Kg. by mouth). 1.0 mg. per Kg. of atropine sulfate by vein. Direct and indirect single shock and tetanic stimulations before physostigmine administration and following the intravenous injection of 0.2 mg. per Kg., 2.0 mg. per Kg., and 3.0 mg. per Kg. of physostigmine.

* Reprinted, with permission, from *Bull. Johns Hopkins Hosp., 83,* 532 (1948).

Bernard reasoned that, since the action of the drug was peripheral and since the nerve and the muscle were still able to function after exposure to the drug, the action of curare must be to block the transmission of the stimulus from nerve to muscle. This established the basic concept back of all studies on neuromuscular transmission. Curare is, therefore, the classic drug for demonstrating blockage. Other drugs do this, and I shall refer to them as curariform agents.

The evidence for the action of drugs which cause or increase neuromuscular transmission is not as neat as the experiments of Claude Bernard. Today, we believe that acetylcholine is the agent which transmits the impulse across the neuromuscular junction; according to the generally accepted theory, it is liberated at the immediate vicinity of the motor end plate when the nerve membrane is depolarized, that it in turn depolarizes the sole plate of the muscle before it is rapidly destroyed by cholinesterase and from this a propagated wave of depolarization spreads over the muscle. But our knowledge of this came slowly and is not yet complete.

In 1921, Loewi (2) established the first proof of the chemical mediation of nerve impulses by the peripheral release of chemical agents. Here, our problem of method of evaluation comes to the fore because technical problems at first prevented acceptance of Loewi's findings. His method was simple: two frog hearts were set up so as to record their activity, and the saline perfusate from one passed on to the second. When the vagus nerve to the first was stimulated, it was slowed or stopped and so was the second after a period long enough for the fluid from the first to reach the second. This showed that vagus stimulation liberated some chemical which affected cardiac muscle. The difficulty came when other workers tried to repeat his work and did not get the same results. Now we know that the vagus is a mixed nerve which contains fibers to accelerate the heart as well as fibers to slow it and that predominance of fiber type varies with the individual frog and with the season of the year.

Earlier, Dale had studied acetylcholine and found that it mimicked the responses obtained when parasympathetic nerves were stimulated. With this evidence in hand it might seem easy to reach proof that acetylcholine was the substance liberated by motor nerves. Here, again, the methods of evaluation of the compound were the stumbling block. Venous blood from muscles, before and after stimulation, simply did not seem to have acetylcholine in it. This

brings two more factors into our historical background—the cholinesterases and drugs which are cholinesterase inhibitors.

The story of cholinesterase inhibitors began with the calabar bean, which was used by West African tribes as an ordeal poison in trials for witchcraft. By 1863, Argyll Robertson had demonstrated the antagonism between it and belladonna on the eye. The chief active alkaloid fraction of the bean is known by the names of physostigmine or eserine. This is, of course, a well-known pharmacological agent which has profound effects on the autonomic nervous system.

Following the discovery of vagus-substance, Loewi and Navratil (3) showed that both acetylcholine and vagus-substance were rendered inactive by heart extracts. The heart extract lost its ability to destroy both substances after it had been heated or treated by ultraviolet

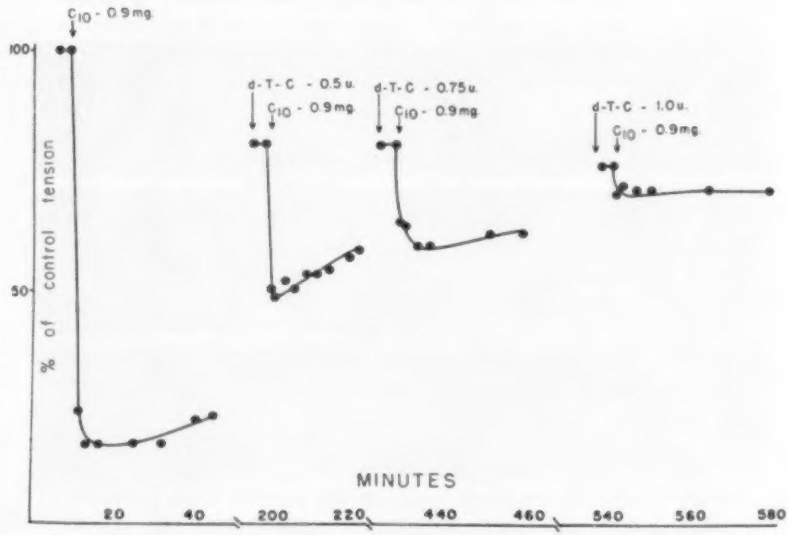

FIG. 2*

Course of C_{10} weakness in triceps surae denervated 14 days. Direct supramaximal stimulation. Strain-gage recording. Progressive inhibition of C_{10} effect by progressively larger doses of D-tubocurarine injected 5 minutes earlier.

*Reprinted, with permission, from *Am. J. Physiol.*, *162*, 475 (1950).

light. This observation inaugurated the study of the enzyme, cholinesterase. In 1930, Englehardt and Loewi (4) found that the addition of physostigmine to blood or tissue extracts prevented the disappearance of added acetylcholine. Thus, physostigmine was shown to be an inhibtor of the enzyme cholinesterase and, therefore, an agent which enhanced the action of acetylcholine by preventing its destruction.

Today, we know that there are a number of different cholinesterases, present in blood and tissues, and that there is a remarkably high concentration of cholinesterase in the region of the neuromuscular junction. For our purpose here, we need only return to the original evidence for the liberation of acetylcholine by motor nerves and evidence as to the effect of cholinesterase inhibitors on neuromuscular transmission.

As mentioned before, it seemed likely from the work of Loewi and others that something like acetylcholine was liberated by voluntary motor nerve endings but the nature and source of the substance was not definite. In 1936, Dale, Feldberg, and Vogt (5) provided definitive evidence; the method they used has sufficient bearing on the problem of the evaluation of drugs affecting neuromuscular transmission to be given in outline.

The first difficulty to be eliminated was that the nerves to mammalian muscles generally have vasodilator fibers in them; since the latter were already known to liberate acetylcholine, it was important to eliminate them. This was done in one type of experiment by using the cat's tongue as a source of muscle and stimulating the hypoglossal nerve; the hypoglossal nerve to the tongue is a purely motor nerve while the vasodilator fibers run in the chorda-lingual nerve. In another type of experiment, the leg muscles of dogs were used and motor stimulation made via the ventral spinal roots after the sympathetic chain had been removed. A second problem was the presence of cholinesterase in blood, which would rapidly destroy any acetylcholine which might be released. This was solved by totally eliminating the blood supply to the muscles being tested and perfusing them with oxygenated Locke's solution at 37° C. To the perfusate, physostigmine was added at a concentration of 2 parts in a million to further protect the acetylcholine. A third problem was to detect and identify the very small amounts of material liberated into a relatively large amount of fluid; the amount of material later

identified as acetylcholine which was collected from the cat's tongue was about 0.06 microgram per minute. Detection was made by measuring the contraction produced in physostigmine-protected leech muscle suspended in isotonic saline; under these conditions, leech muscle responded well to acetylcholine in a dilution of one part in a million and is a more sensitive indicator than a chemical method. Identification was made by testing the material on the blood pressure of the cat and comparing the relative effects on leech muscle and cat blood pressure with known solutions of acetylcholine. Also, it was shown that the material liberated by stimulation of the motor nerves was protected from destruction by physostigmine, as is acetylcholine. Further, it was shown that the effect of the material on the cat's blood pressure was blocked by atropine. Finally, the material was found to be acid stable but rapidly destroyed in dilute alkali, as is acetylcholine. The authors concluded that there were no grounds for doubting that the material liberated by motor nerves was acetylcholine

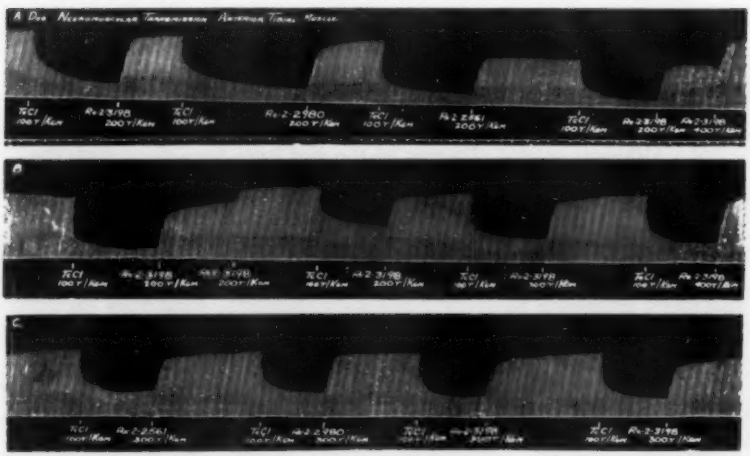

FIG. 3*

Anticurare action of phenolic quaternary ammonium salts on neuromuscular transmission. Dog 10.0 Kg. Dial-urethan anesthesia. Contractions of the anterior tibial muscle. Time in minutes. Stimulation of the peripheral end of the sciatic nerve, 6.7 times per minute. Injection into the femoral vein.

* Reprinted, with permission, from *J. Pharmacol. Exptl. Therap.*, *100*, 83 (1950).

itself. They also showed that curare did not stop the release of acetylcholine but that, when the motor nerves to a muscle were cut and had degenerated or when the nerves were stimulated to the point of exhaustion, no acetylcholine was liberated.

The next step was to show how physostigmine, with its known ability to protect acetylcholine from destruction and enhance muscular contractile force, acted on muscle. The details of this were reported by Brown (6) in 1937. He recorded the electrical action potentials of mammalian muscles which were stimulated by a single maximal shock to their nerve. Before physostigmine, the muscle responded to a single volley of nerve impulses with a single twitch and a single electrical response. Within 5 or 10 minutes after the intravenous injection of physostigmine, the mechanical response had reached maximal potentiation, and the muscular response to a single nerve shock consisted of repetitive discharges. The author concluded that the effect of physostigmine on muscle was mediated through its anticholinesterase activity, allowing acetylcholine to persist at the end plate in a supraliminal concentration which in turn initiated repetitive discharges from the region of the motor end plate. A number of authors have shown that, while small doses of physostigmine potentiate the effect of nerve stimulation or acetylcholine on muscle, large doses prevent rather than enhance these effects.

Although several concepts or theories have been developed, the chemical concept of synaptic transmission as recently developed by Grundfest (7) seems to fit the evidence better than any other. According to this, nerve impulses are conducted along the nerve axon as all-or-none responses and set up a graded response at the end plate with secretion of acetylcholine. Acetylcholine is the chemical transmitter, which causes its effect by depolarization of the post-synaptic membrane of the muscle cell. Since the response at the end plate is graded according to the amount of acetylcholine, the duration of the response may be altered by varying the stimulus in rate or duration, or by varying the rate of destruction of acetylcholine. Sustained application of the depolarizing drug causes desensitization of the chemically excitable membrane, and this is probably a relatively common physiological phenomenon. On this concept, we would speak of two types of drugs which affect neuromuscular transmission—*synapse activators* and *synapse inactivators*. The synapse activators would be those which depolarize the membrane, i.e., acetylcholine or its

mimetics; synapse inactivators, of which curare is an example, would be those which diminish or eliminate synaptic electrogenesis. It is interesting that decamethonium and succinylcholine would be classed here as synapse activators since they depolarize the synapse; their curare-like activity is apparently due to overwhelming depolarization of the membrane.

From here on, I shall refer to only three classes of agents (I) curare and curariform drugs, which inhibit neuromuscular transmission; examples of these are d-tubocurarine chloride and dimethyltubocurarine (Mecostrin® or Metubine®) chloride or iodide and gallamine triethiodide (Flaxedil®) in the blocking group, with decamethonium (Syncurine®, C10), benzquinonium chloride (Mytolon®), and succinylcholine (Sucostrin®, Anectine®) chloride in the depolarizing group; (II) the natural transmitter acetylcholine and drugs which mimic it, and (III) cholinesterase inhibitors, such as physostigmine or eserine, neostigmine (Prostigmin®) bromide or methylsulfate, pyridostigmin (Mestinon®) bromide, and di-isopropylfluorophosphate (Floropryl®, DFP). No mention will be made of atropine, pilocarpine, or agents which act principally on neuromuscular transmission to smooth muscle, nor of central relaxants such as mephenesin or meprobamate though their effects resemble those of the curare-like agents, nor of choline acetylase since I am not aware of any drugs which are known to act on this enzyme and thus affect neuromuscular transmission.

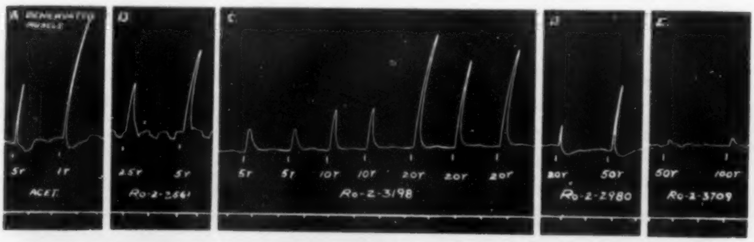

FIG. 4*

Stimulating action on denervated muscle. Dog, 14.0 Kg., chloralose anesthesia. Denervated eleven days prior to the experiment. Contractions of the anterior tibial muscle. Time in minutes. Injections into branch of the femoral artery.

* Reprinted, with permission, from *J. Pharmacol. Exptl. Therap.*, *100*, 83 (1950).

Evaluation of Curare and Curariform Drugs

The laboratory assay of curare-like drugs is usually done by the rabbit head-drop method, which was described fully by Varney, Linegar, and Holaday (9) of Squibb in 1949. Their paper is a classic, and I shall quote from it extensively.

"In selecting a suitable method for the assay of curare preparations, several procedures have been investigated and evaluated. The method of finding the minimum dose of curare required to produce paralysis in frogs following injection into the ventral lymph sac was found impractical because of the very large number of animals necessary to obtain a precise evaluation of relative activity of test sample and standard preparation. Determination of the lethal dose by subcutaneous injection into mice and other animals has also been used as a method for standardizing curare extracts. Our results when using the subcutaneous LD_{50} for evaluation of potency indicated that the same objections apply to it as to the frog paralysis test.

"The determination of the intravenous LD_{50} (50 per cent anoxic fatal dose) in three species was investigated as another approach to this problem. Although this procedure proved fairly accurate in the rabbit, it was considered too costly for routine assays. In comparison with the results obtained in rabbits, those in mice and particularly those in rats showed much greater variation in response to a single rapid injection.

"Concurrently with these studies the curare effect on local muscle groups was investigated as a possible assay procedure." The Squibb workers continue to discuss these methods, point out the accuracy of some methods but conclude, "Unfortunately it was found that some curare preparations, even from the same source, purified by the same procedure and standardized by the dog gastrocnemius method, showed widely different activities when used in humans."

They, therefore, developed the method of assay in the intact, unanesthetized animal. Though the endpoint was sharp and constant from day to day in dogs, cats, and monkeys, these animals were more difficult to handle and more expensive than rabbits. "Preliminary investigations of the parenteral modes of administration of curare showed that the intravenous route gave the most constant and reproducible results since the curare effect was fairly rapid in onset and of comparatively short duration." The end point of the assay

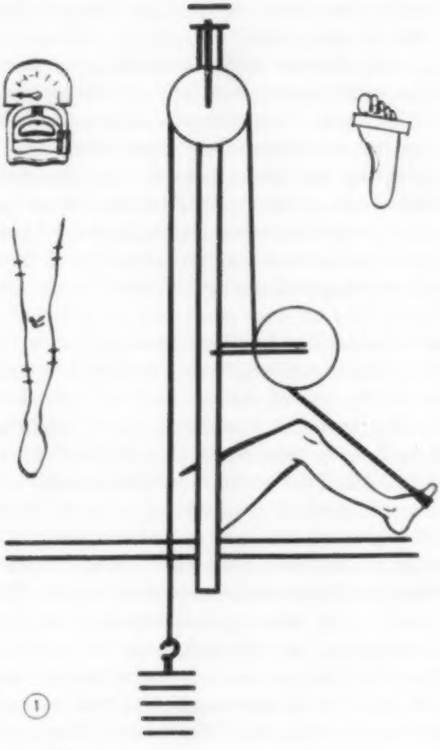

FIG. 5*

Method of dorsiflexing the feet under graded levels of tension. Adding successive weights dorsiflexed the foot to effect graded levels of tension in the gastrocnemius. The rope tugged parallel to the longitudinal axis of the shin. Bipolar electrodes were placed on the peroneal, gastrocnemius, quadriceps, and hamstring muscles in both legs. Immobilizing the insulated electrodes to the skin prevented artifacts from appearing in the records, even with severe clonus. Stirrup placement was standardized, with the edge of the strap along the skin fold at the base of the big toe and the little toe. Hand grip fatigue studies were performed concurrently.

* Reprinted, with permission, from *Neurology, 8,* 446 (1958).

was clear—it "is the precise relaxation state when the animal's head falls to the board and cannot be raised or turned in response to a light tap on the animal's back."

Having found a practical animal, route of injection, and end point, the authors proceeded to study carefully all the factors which might cause variation of results. One of these was the rate of administration. "When the material was injected too slowly, the administration rate so closely approached the elimination rate that the end point either was not reached or was achieved only after administration of relatively large amounts of the drug over an unduly prolonged period. On the other hand when the administration was too rapid the full effect of the amount of curare given was not obtained at the time head-drop was reached, and later an over effect was produced as evidenced by prolonged curarization and increased incidence of respiratory paralysis." An intermediate rate was found adequate—this consisted of the injection into an ear vein of 0.1 cc. every 15 seconds of a solution containing 2 units per cc. of a standard curare solution. End point was reached in 3 to 8 minutes by this method; that is, after the injection of 1.2 to 3.2 of the arbitrary units of curare.

The reference standard consisted of a stable desiccated curare powder stored to prevent change in moisture content. One milligram of this was taken as one unit; when pure d-tubocurarine chloride was prepared, it was found to have one unit of activity in 0.155 mg.

Further studies on other factors which might cause variation showed that differences in body weight of the rabbit were not important. The effect of previous doses of curare were, however, important. Though the animals seemed to recover completely from a single head-drop dose in about 10 minutes, doses given up to 4½ hours later were found to be more effective. Therefore, a rabbit could be used only once a day. Another factor was the need for preliminary injections. Strangely, the dose necessary to produce head drop shows less variation after the animals have had two or more daily injections within the previous 5 days.

To measure the potency of an unknown solution of material, a rough estimate of its potency is first made and a solution made up which contains about 2 units per cc. plus or minus 10 per cent. For the actual test, a standard solution of curare is given to at least 8 rabbits and the test solution to an equal number. The dose necessary to cause head drop in each rabbit is noted. The procedure is re-

peated the next day, on the same rabbits, with a cross-over pattern; that is, the rabbits which had been given the test solution the first day are given the standard solution the second day, and vice versa. In all cases, the rate of injection is 0.1 cc. each 15 seconds, delivered by gravity from a 5 cc. microburette through small bore tubing connected to a needle in an ear vein of the rabbit.

To calculate the potency of the test solution, the ratio of the volume of the standard to the test solution to produce head drop is calculated for each rabbit. "The geometric mean of these ratios together with its standard error is then calculated; this mean multiplied by the dilution of the test solution and by 2 (potency of standard in units per cc.) equals the potency of the undiluted sample in units per cc."

This method has become the official method for laboratory assay and is both accurate and highly reproducible. It is, however, an animal test and we know that marked species differences in the susceptibility to drugs exist. For example, dimethyl d-tubocurarine is about 10 times as potent as d-tubocurarine when assayed in rabbits, but less potent when tested on mice. Therefore, evaluation on man is essential.

Unna *et al.* published two papers (10, 11) titled, "Evaluation of Curarizing Drugs in Man." The work was carefully thought out, and they studied the effects of d-tubocurarine, dimethyl tubocurarine, and decamethonium on unanethetized normal men. Essentially, they measured the effects on respiration and grip strength while the three curariform agents were injected intravenously over a 90-second period, at regular intervals afterwards until there was return to normal, and after a second injection to determine whether the effects of the first had worn off. This simple sentence does not do justice to their study. Full equipment for intubation, oxygen mask, and respirator bag were in constant readiness in case of respiratory embarrassment, though they were never needed. Spirometer measurements of respiration were made continuously, as were pneumographs of the intercostal and diaphragmatic components of breathing. Ophthalmological observations were made, as well as electrocardiograms, electroencephalograms, blood pressure records, a watch for fasciculations, tests on pain threshold, and subjective records as to vision, order of muscle weakness, etc.

The dose of the drug needed to produce 95 per cent loss of grip strength (the GD_{95}), expressed as micrograms of ionized drug per kilogram of body weight, was calculated from the records on the dynamometer. The time to reach maximal curarization was noted, as well as the time to recovery which was taken as the point at which the grip strength had returned to 75 per cent of the baseline. Since there were comparable measurements of these variables on all three drugs, it was possible to make a *relative evaluation* of the three different drugs as to the following points:

(a) The dose needed to produce 95 per cent grip paralysis.

(b) The duration of grip paralysis.

(c) The depression of vital capacity.

(d) The order of onset of weakness in muscles and order of recovery.

(e) The relative effects of the drugs on ocular mobility.

(f) The presence or absence of muscle fasciculations.

(g) The effects on sensations, which were none except those expected from muscle weakness.

(h) The persistence of the drugs in the body after recovery of grip strength.

(i) The relative safety of the different drugs in which they defined the "coefficient of safety" as the relationship between decrease in grip strength and depression of vital capacity.

(j) The predictability of a given dose.

It is not the purpose of this paper to review their results, but it is worth noting that they observed tachyphylaxis to C_{10} in humans though it could not be observed in animals, that the "respiration sparing" effect of C_{10} observed in cats and monkeys could not be found in man, and that relative potency of the drugs in man was different from that in rabbits. Their final sentence reads: "Prediction of quantitative responses in man, on the basis of experiments performed in animals, is fraught with danger."

The action and interaction of the two types of curariform drugs was evaluated by Jarcho and his collaborators (12). They used d-tubocurarine as an example of the blocking type and decamethonium as the depolarizing type. They used the anterior gracilis muscle of the rat, which can be easily prepared for surface recording without disturbance to its circulation. Muscle tension was recorded by a strain gauge; stimulation to the nerve and directly to the muscle was made electrically; action potentials from nerve and muscle were recorded electromyographically. The demarcation potential of the muscle was measured between the intact proximal third of the muscle and a crushed or burned area in the distal area. End plate potentials were recorded by freeing the muscle from overlying tissue and exploring the muscle after neuromuscular block had been produced. The end plate potentials were readily found in a localized zone about 3 mm. wide in the proximal third of the muscle near the nerve entry. Studies were also made after the muscle had been denervated.

Their work confirmed the production of complete neuromuscular block by both drugs. Both caused a drop in amplitude of the end plate potential and decrease in size of the propagated muscle action potential. The depression occurred without change in the action potential of the nerve or the ability of the muscle to respond to direct stimulation.

There were, however, marked differences in other actions of the two agents. Decamethonium caused a prompt fall in the demarcation potential of the innervated and denervated muscle, which was blocked by d-tubocurarine. It also reduced the contraction strength of directly stimulated denervated muscle, an action which was blocked by d-tubocurarine. They concluded that both drugs were active both at the end plate and along the muscle fiber.

Evaluation of Muscle Stimulant Drugs

The evaluation of acetylcholine, the normal mediator of transmission at the neuromuscular junction, is included because of its key position and pharmacologic interest though it is not a drug for general human use. Points of key interest are, of course, the need for protecting acetylcholine from destruction by a cholinesterase inhibitor and the technique of measuring the very small amounts of acetylcholine which are usually too small for chemical analysis.

The dorsal muscle of the leech has been used for a long time for the estimation of acetylcholine. In this method, the muscle is placed in a bath of saline which is as small as practical (about 2 ml.) and its contraction recorded on a kymograph. Fluid containing known concentrations of acetylcholine is added to the bath to show the responses to known amounts, and unknown solutions are compared with these responses. The advantages are the cheapness and relative ease of the preparation, which is sensitive in the range of a few nanograms * of acetylcholine. The disadvantages are the need for dilution of the samples and the tendency for the muscle to change sensitivity so that repeated calibrations are needed. Physostigmine (eserine) should be in the diluent fluid to a concentration of about 1 part in 3×10^5, and identification of the acetylcholine made by methods outlined above as well as blocking of contraction by d-tubocurarine.

The conventional bioassay of acetylcholine is made by injection of the unknown material into an eserinized anesthetized cat, the effect on blood pressure recorded by manometer, and comparisons made with known solutions. Recently, Straughan (13) has described a method for the bioassay of acetylcholine on the rat blood pressure. "By using sodium pentobarbitone with urethane as the anesthetic and allowing the body temperature to fall to about 28°, it has proved possible to get a long-surviving stable sensitive preparation." This author used neostigmine as a cholinesterase inhibitor, reported the dead space of the injection system to be only 0.03 ml. and the preparation to be stable for hours and sensitive to doses of acetylcholine as low as 0.5 mg.

Acetylcholine has rarely been used in man since it is so quickly destroyed. For special studies, however, it may be used by direct intra-arterial injection for the evaluation of the patient. Grob, Johns, and Harvey (14) used it to show the difference in the response between normal and myasthenic patients.

Edrophonium, usually referred to by its trade name of Tensilon ®, is a little known drug which is of considerable interest. It is apparently both a direct muscle stimulant and an anticholinesterase. It was discovered by Randall (15) in a search for anticurare agents and is chemically related to neostigmine. Randall's method will be given in some detail, since it is one used for evaluation of cholinesterase inhibitors.

* A nanogram in one-thousandth of a microgram, or 0.000001 milligram.

Twenty-seven new compounds were evaluated for their effect on the curarized anterior tibial muscle of the chloralose-anesthetized dog, stimulated via its nerve. The animals were given 1 mg./Kg. of atropine to prevent circulatory effects of the drugs and enough d-tubocurarine chloride intravenously (usually 0.075 to 0.15 mg./Kg.) to produce nearly complete neuromuscular block. Once the block was established, the new agent was given intravenously.

Potentiation of the muscle response to indirect stimulation was made similarly. The animal was prepared as before to record twitches induced by single maximal shocks to the peripheral end of the sciatic nerve, but the animal was not curarized. The compounds were injected into the artery leading to the muscle in graduated doses and their potentiation of muscle response noted.

The effect of the drugs on denervated muscle proved the clue to the stimulant effect of edrophonium. The sciatic nerve to one leg was cut 10 to 14 days before the experiment. The dogs were anesthetized with Dial ®-urethane, the anterior tibial muscle connected to the lever for recording, and the compounds injected intra-arterially. Acetylcholine was injected first to check on the response of the muscle; the new compound produced contractile action also in denervated muscle.

Further evaluations were as follows: The anticholinesterase effect of edrophonium was measured by the manometric method of Ammon (8) and found to be only one-hundredth that of neostigmine, though its anticurare effect was one-quarter that of neostigmine. Further, the effect of the drug on stimulation of the isolated intestine was only one-tenth that of neostigmine. Thus, it seemed clear that Tensilon ® had some anticholinesterase activity but that it also had direct muscle stimulant activity.

This last view has been questioned by Hobbiger (16), who studied the mechanism of anticurare action of neostigmine analogs. He evaluated them in muscle preparations in which the cholinesterase activity had been totally blocked by D F P. In this preparation, he found that acetylcholine, choline, and decamethonium still maintained anticurare action; whereas, Tensilon ® lost this property. There the matter rests at the moment, with Randall's data indicating direct muscle stimulation to be the chief mode of action from effects on denervated muscle and Hobbinger's data indicating anticholinesterase activity to be the mode of action from studies on D F P poisoned muscle.

Fortunately, the method of evaluation of Tensilon® on human beings is simple. If a *normal* man is given 10 mg. of the drug by rapid intravenous injection, results are minimal but prompt; skeletal muscle fasciculations will be noted which last only a few minutes. Changes in blood pressure, heart rate, and muscle strength are minimal though there may be mild brief symptoms such as blurred vision, perspiration, dizziness, faintness, or warmth.

The diagnostic use of Tensilon® in myasthenia gravis should probably be referred to as an evaluation of the patient, rather than of the drug, and is valuable. The injection of 10 mg. of Tensilon® intravenously to a patient with myasthenia gravis quickly increases muscle strength, usually without causing fasciculations. In the few patients with this disease to whom I have given Tensilon®, the usual first effect noted was their grateful comment, "Oh, doctor—I am much better." For reasons unknown to me, the mild undesired effects of the drug are usually lacking in such patients and the increase in muscle grip strength can be measured by a dynamometer.

Another clinical use of Tensilon® is as a *curare antagonist* in anesthetized patients. Here, the effect of the drug is best evaluated by its effect on respiration. It is interesting that, though the effect of the drug is so transient in normal persons, it appears to maintain decurarization when that has been established. (Of course, it is not an antagonist to succinylcholine or decamethonium.)

Evaluation of Cholinesterase Inhibitors

Probably, the most reliable method for screening evaluation of new cholinesterase inhibitors, as well as comparative evaluation of anticholinesterase activity of known compounds, is the *in vitro* method of Ammon (8) or its modifications, using the Warburg manometric technique. Those interested in the several methods available, as well as the details of their procedures, should refer to the review by Augustinsson (17) titled "Assay Methods for Cholinesterases."

In brief, the method in the Warburg apparatus is as follows: A standard buffered enzyme solution is added to the main compartment of the flask and a standard amount of substrate (acetylcholine itself, the d-isomer of acetyl-β-methyl choline or other) is added to the side bulb. Nitrogen with 5% CO_2 is run through the apparatus which is then closed and allowed to come to temperature equilibrium

in a water bath, and the first manometer read. The substrate is then added to the enzyme at zero time, and at the amount of CO_2 produced read at intervals. The amount of CO_2 is plotted against time in minutes and used as a measure of enzyme activity. Unmixed controls are used to measure the spontaneous hydrolysis, which is subtracted from the observed values.

In testing anticholinesterase compounds, the agents are included in the solution with the enzyme so that it will have time to act before the substrate is added. Different concentrations of the agents are used, and results usually expressed in terms of the concentration needed to produce 50 per cent inhibition of enzyme activity. A convenient check is to include a known potent cholinesterase inhibitor such as neostigmine in the study.

Though the *in vitro* method gives a good measure of anticholinesterase activity, it must be clearly noted that it does not indicate clinical activity. Neostigmine inhibits purified electric eel esterase to about 80 per cent when diluted to 5×10^{-5}M. Tensilon® is only one-hundredth as potent *in vitro*, but is nearly as active clinically. It is curious that decamethonium has been reported to have anticholinesterase activity too, though it is weak.

The evaluation of cholinesterase inhibitors in laboratory animals may be carried out by testing their ability to potentiate the muscle response to indirect stimulation, as anticurare agents and also as direct muscle stimulants. The method was outlined for Tensilon®. As Randall and Jampolsky (18) have pointed out, anticurare activity and anticholinesterase activity are not the same.

Tested by these methods, physostigmine shows *in vitro* anticholinesterase activity, ability to potentiate muscle responses in low dosage and to depress it in high dosage, good anticurare activity, but no ability to stimulate denervated muscle. Neostigmine has similar properties but also has some ability to stimulate denervated muscle according to Randall, though it lacks the ability to stimulate muscle poisoned by D F P according to Hobbinger. This apparent discrepancy is as yet unexplained, though they may eventually be explained on the basis of multiple actions by D F P. Pyridostigmin is similar to neostigmine in animal tests, though it is less potent on a weight basis.

No more than mention will be made here of di-isopropyl-fluorophosphate (D F P), one of the group of phosphates found to cause

permanent injury to cholinesterase. Its story belongs with that of war gases and insecticides, and its chief clinical use is in glaucoma.

The evaluation of cholinesterase inhibitors in man is quite different from the laboratory. The important points to remember are that they inactivate the enzyme in many parts of the body and may cause weakness due to overdosage as well as from inadequate effect.

An example of clinical evaluation is that done by Osserman (19) with Mestinon ® for myasthenia gravis. Knowing that the new drug had properties similar to Prostigmin ®, he gave patients a dose equivalent to their previous medication and attempted to answer four questions. The first was: "What is the duration of the drug?" The method of evaluation for this was the serial Tensilon ® test, in which the patient measures grip strength by a dynamometer or pulls an ergograph in time with a bell which rings at regular intervals. Tensilon ®, in a dose of 0.2 cc. is given intravenously; improvement in strength indicates that the patient is underdosed; increasing weakness from the Tensilon ® indicates the patient is overdosed due to cholinergic depolarization. The second question was: "What relief from myasthenic symptoms does it offer?" This was answered by the patients' replies. The third question was: "Is the drug non-toxic?" For this, he performed routine physical examinations and took serial x-rays of the chest to see if there was enlargement of the thymus, serial electrocardiograms, tests of thyroid activity, urine examinations, blood counts, and bone marrow examinations. He also compared the patient's number of gastro-intestinal symptoms on the previous drug with the new, including their need for atropine. The final question was: "Can the drug be helpful when the patient is resistant to neostigmine?" This, he answered by trying it in resistant cases. In addition, he tried the drug in combination with others to see if there was synergism. He also noted the characteristic signs of overdosage with the new drug.

Robert Schwab of Boston studied the same drug. He asked the same questions, used repeated tests with the dynamometer as his chief measure, and came to similar conclusions with a different method of evaluation.

Curare and prostigmine have been used for the evaluation of the myasthenic. They are inferior to Tensilon ®—the first makes the patient worse, and the second may cause prolonged undesirable effects.

The Selection of Drug for Clinical Use

This final section, to which I have applied the heading "The Selection of Drug for Clinical Use" might be entitled "The Doctor's Evaluation of the Drug." It is intentionally made a separate section, for there is many a slip twixt the Warburg apparatus and the patient's improvement. One of these slips is the difference between results obtained in animals and that in man, as pointed out repeatedly. Another is that a drug has more than one effect in the body, and the so-called side effects may be important. As a result, a drug which appears excellent in animal tests may have disadvantages in man, while a drug which seems to have disadvantages may be really quite practical.

Probably, the most popular curariform drug is succinylcholine, despite the facts that it sometimes induces cardiac arrhythmias, may cause persistent respiratory depression, and there is no consistent and safe antagonist to it. Succinylcholine is usually used by anesthesiologists who may give it by constant drip during an operation; they like its excellent relaxant effect. Because Pentothal ® reduces the incidence of cardiac irregularities produced by succinylcholine and is usually used with it and because anesthesiologists are experts at artificial respiration and have the equipment for it available beside them, they do not worry about these disadvantages. Psychiatrists use succinylcholine during electroshock therapy, and they like its short action. They also use Pentothal ® and have respiratory help available. Gallamine and d-tubocurarine appear to be safer, since they are antagonized by neostigmine and edrophonium. But gallamine has a vagolytic action and, therefore, should not be used in a patient in whom tachycardia is undesirable. Similarly, d-tubocurarine may release histamine and must be used with caution in patients with a history of allergy and in patients in whom a fall in blood pressure might be dangerous.

There is a need for muscle relaxants for the treatment of spastic diseases such as occur in tension states, following injury, or in cerebral palsy. But clinical evaluation of the curare drugs finds them wanting. Their short action, the fact that respiratory muscles are paralyzed along with other muscles and that they must be given parenterally makes them impractical.

The centrally acting muscle relaxants such as mephenesin and carisoprodol (Soma ®) have proved practical since they can be given

without causing significant respiratory depression. It is worth noting here that the evaluation of such muscle relaxants in humans is difficult and largely dependent on palpation for spasticity and tenderness. Stimulation of muscles to the hand via the ulnar nerve, as described by Botelho (20), with recording of the muscle tension and electric response is a practical approach for their evaluation. Electromyography is mentioned here only to regret its present unsatisfactory state. Hope for the future, however, is indicated in the work of two Philadelphia workers, one of whom is Vazuka of Temple. He has devised a method (21) which uses a standardized technique of stretching the gastrocnemius muscle at graded levels of tension and recording electromyographic responses. His method is briefly as follows:

"Bipolar surface electrodes were placed at standardized positions over the belly of the peroneal, gastrocnemius, quadriceps, and hamstring muscles of each lower extremity. The leads were connected through a Grass machine differential amplifier to the multichannel ink-writing apparatus . . . ." Stirrup placement across the sole of the foot was standardized—a "rope attached to the stirrup dorsiflexed the foot . . ." and was weighted to effect various degrees of tension. The patella and Achilles tendons were stimulated by a standard blow of a reflex hammer. Action potentials from the eight muscles were recorded simultaneously. Action potentials from the responding gastrocnemius under graded tensions serve as simple and reliable indicators of reflex activity. Alterations in severity of ankle clonus and in severity of response to sensory stimuli are readily apparent and amenable to statistical analysis. By this method, Vazuka was able to compare the effects of several drugs. The method appears to represent the proper approach though it is not yet standardized completely. Its greatest use would be in spastic diseases.

Erdman and Heather, of the Hospital of the University of Pennsylvania, have published (22) a preliminary note on "A method for quantitative measurement of spasticity and its response to therapy." Though the drugs studied by these workers are central relaxants, I see no reason why their methods cannot be applied to the evaluation of drugs acting on the neuromuscular junction. Future reports are eagerly awaited.

The clinical evaluations of direct muscle stimulants and of cholinesterase inhibitors are similar. They depend on the relative proportion of desirable and undesirable results, as well as the speed of action

and duration. Edrophonium® is the drug of choice for testing purposes, where its prompt brief action and minimal autonomic effects recommend it. As an antagonist to the synapse stabilizing curare drugs, it is prompt and the results long lasting if an adequate amount is given. For their action on the neuromuscular junction, the use of these drugs is largely confined to myasthenia gravis. Though physostigmine is effective in this condition, it penetrates the blood brain barrier and produces undesirable mental symptoms. Therefore, until recently, neostigmine was the drug of choice for myasthenia even though it produces epigastric distress, abdominal cramps, and diarrhea. As noted above, pyridostigmine has been found superior. Though only one quarter as effective on a weight basis, its effect is longer lasting and a dose which is equally effective on muscle strength causes less gastrointestinal disturbances. Many patients who were maintained on neostigmine plus atropine to block the autonomic effects can be maintained on pyridostigmine alone. Curiously, though D F P would seem the ideal anticholinesterase by laboratory studies (because of its long effect) its autonomic effects are so overwhelming in doses which enhance muscle strength that it has not proved practical.

And, finally, I must note that there is a paper (23) reporting the therapeutic use of d-tubocurarine in myasthenic crisis. The basis of this apparent impossibility was that the patient was needing larger and larger doses of neostigmine but rest of the muscles seemed to help her. "Large doses of d-tubocurarine, which provided complete rest for the motor end plates, led to a dramatic improvement which was sustained with the aid of reduced neostigmine therapy . . . ." Truly, the evaluation of drugs on the patient is different from their evaluation in the laboratory.

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A DECADE OF CHEMOTHERAPEUTIC MANAGEMENT OF CHRONIC GRANULOCYTIC AND CHRONIC LYMPHOCYTIC LEUKEMIA

By John R. Sampey *

CHRONIC granulocytic and chronic lymphocytic leukemia are the two most common types of chronic leukemia. The term "chronic myelocytic leukemia" is used more frequently in the literature than "chronic granulocytic leukemia" for, by actual count in this report, the term "myelocytic" is employed 3577 times and "granulocytic," in only 1108 cases of chronic leukemia. However, the term "granulocytic" has been recommended when referring to a disease affecting any cell of the granulocytic series in the "1949 Recommendations of the Committee for Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood-forming Organs" (*Blood 4,* 89-96, 1949). The Committee recommends that terms such as "myeloid," "myelogenous," and "myelocytic" leukemia be avoided. Moreover, Blakiston's *New Gould Medical Dictionary,* 2nd ed., 1956, refers from "Leukemia, Myelocytic" to "Granulocytic Leukemia", and John Crerar Library in preparation of *Leukemia Abstracts* substitutes the term "granulocytic" for "myelocytic" (Personal communication).

A recent study of the chemotherapeutic management of acute leukemia (25) disclosed that folic acid antagonists, ACTH/adrenal steroids, and 6-mercaptopurine were the most frequently employed chemicals during the last decade. None of these three agents is found among the three leading chemicals for the control of chronic leukemias in this study. This paper will bring out also several challenging differences in the tabulations on more than 7500 patients with the two most prevalent forms of chronic leukemia.

Chronic Granulocytic Leukemia

Myleran, radiophosphorus, and nitrogen mustards account for two-thirds of the patients with chronic granulocytic leukemia who received chemotherapy in the decade covered by this review. Current reviews (23) in the literature lend interesting statments of corrobora-

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tion and modification of this conclusion. Myleran is the agent of choice in a dozen current reviews (4-6, 9-13, 19, 24, 26, 27).

The AMA Council Report (2) in 1956 concluded that x-rays and P^{32} were the best therapy, and that myleran and triethylene melamine (TEM) were useful in chronic granulocytic leukemia. Bove and Emerson (8) considered myleran and N-mustards the agents of choice. Burchenal and Krakoff (10), and Galton (17) found myleran, colcemid, and 6-mercaptopurine (6-MP) the best agents. Diamond (14) selected myleran as the agent of choice, and he considered 6-MP especially effective in the later stages of granulocytic leukemia. Ellison and Burchenal (15) favored the following order of therapy: myleran, TEM, phosphoramides, HN2, etc. Best and Limaryl (7) indicated their order of preference by giving 4 + marks for therapy with x-rays and P^{32}, 3 + for urethan and myleran, and 1 + for mustards, TEM, and 6-MP. Larionov (20) rated HN2, TEM, and myleran with 2 + marks each. Amado Ledo (3) and Marlow and Bartlett (21) rate P^{32} as the agent of choice, and Brimi (9) and Fowler and Jolly (16) give the radioisotope high priority in the management of chronic granulocytic leukemia. Wintrobe, *et al.*, (27) list HN2, TEM, and folic acid antagonists and cortisone as contraindicated. Ariens (4), Burchenal and Ellison (11), Diamond (14), and Schilten and Pribilla (26) conclude that arsenic is useful in the clinical control of chronic granulocytic leukemia. And finally, a panel (22) concluded that myleran was one of the most useful drugs in this form of leukemia.

Table I presents tabulations from several hundred clinical reports on the management of chronic granulocytic leukemia with chemicals. Column 3, "No. Not Evaluated", designates the number of patients whose response to the various drugs could not be determined in the published reports. In grouping the patients into the two classes, "Good Remissions" and "Fair Remissions" (Columns 4 and 5), three factors in the response were considered, namely, (1) clinical improvement, ranging from subjective response to complete absence of symptoms of the disease; (2) the hematologic response, as reflected in blood counts, and bone marrow; (3) duration of the induced effects of the therapy.

The high percentages of good responses in Table I with eight of the ten agents scoring 72 per cent or better in remission rates is one of the striking features of the data. The large number of patients

whose responses are not indicated in the reports on P^{32}, urethan, and N-mustards, however, limits the significance of this conclusion. The high ratio of good to fair responses in myleran is another feature of Table I.

TABLE I

MANAGEMENT OF 4685 CASES OF CHRONIC GRANULOCYTIC LEUKEMIA

Chemicals	*Total Cases*	*No. Not Evaluated*	*Good Rems.*	*Fair Rems.*	*Remission Rate*
Myleran	1265	140	738	209	84%
P-32	1071	329	454	119	77%
N-mustards	673	253	163	168	79%
Urethan	620	274	101	149	72%
TEM	516	20	195	164	72%
6-MP	192	27	63	91	93%
Colchicines	174	6	98	60	94%
FAA*	93	3	30	24	60%
ACTH/cortisone	73	10	26	27	84%
Antibiotics	8	—	1	3	—

* FAA = folic acid antagonists.

Chronic Lymphocytic Leukemia

Radiophosphorus, N-mustards, and triethylene melamine dominate the therapy of chronic lymphocytic leukemia. It is interesting to note that two of these agents are also most frequently used in the therapy of chronic granulocytic leukemia. Current reviews, however, reflect the confusion which exists among clinicians on the management of chronic lymphoid leukemia. Amado Ledo (3), Best and Limarzl (7), Brimi (9), Fowler and Jolly (16), and Marlow and Bartlett (21) consider x-rays and P^{32} as the agent of choice. Wintrobe, *et al.* (27), place TEM on a par with P^{32}; Ariens (4) and Schulten and Pribilla (26) prefer TEM. Chatterjea (12), Ellison and Burchenal (15), and Karnofsky (18) include TEM among the best drugs for chronic lymphocytic leukemia. Amado Ledo (3) lists arsenic ahead of TEM, and Bernard (6) gives 3 + marks for the N-mustard, CB1348, and only 1 + mark for TEM. Ariens rates TEM 3 +, N-mustard and As 2 + each, and urethan and cortisone 1 + each. Davis accords x-rays first place in the control of chronic lymphocytic

leukemia, and N-mustards second place. CB1348, HN2, and the ethyleneimine, E39, is the order of preference of Lachapelle and Hirtz (19). Galton concluded that chronic lymphocytic leukemia was not as responsive as chronic granulocytic leukemia to chemotherapy.

Table II tabulates the same ten most frequently used drugs in the management of chronic lymphocytic leukemia. P^{32} ranks first in the total number of patients treated, and the ratio of good to fair remissions is high, but the results on one-third of the patients could not be evaluated from the published data, and a remission rate of 59% is low, compared to 85% for ACTH/cortisone and 77% for both N-mustards and urethan. Myleran dropped from first place in chronic granulocytic to a poor sixth place in lymphocytic leukemia therapy. On the other hand, ACTH is very effective in lymphocytic but it makes a poor showing in granulocytic leukemia. Another interesting conclusion from a comparison of data in Tables I and II is that five drugs have similar action in the two tabulations, but that two with the highest remission rates in granulocytic show none in lymphocytic leukemia.

TABLE II

MANAGEMENT OF 2856 CASES OF CHRONIC LYMPHOCYTIC LEUKEMIA

Chemicals	*Total Cases*	*No. Not Evaluated*	*Good Rems.*	*Fair Rems.*	*Remission Rate*
P-32	896	270	414	57	59%
N-mustards	762	80	271	256	77%
TEM	704	91	289	151	71%
ACTH/cortisone	209	29	55	99	85%
Urethan	133	33	53	24	77%
Myleran	66	—	24	8	48%
Colchicines	53	35	6	1	—
Antibiotics	26	—	5	3	—
FAA	7	—	—	3	—
6-MP	—	—	—	—	—

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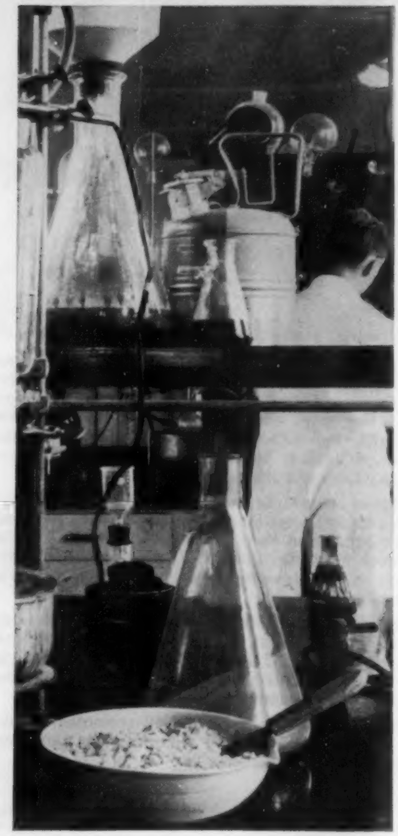

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